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(54) **Method of preparing enzyme conjugates, their use and coupling agents for use in the method**

(57) Enzyme conjugates useful in immunoassay methods are prepared by reacting a coupling reagent having the formula

$X\text{-}(CH_2)_n\,COR$

wherein X is halogen; n is from 1 to 8 and R is a radical which reacts with the amino group of the macromolecule, firstly with an amino-containing macromolecule, and thereafter with a enzyme comprising a sulphhydryl group. The enzyme conjugates can be prepared by this method in a high yield and of high specificity.

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SPECIFICATION

Method of preparing enzyme conjugates, their use and coupling agents for use in the method

Enzyme-macromolecule conjugates are typically used for the detection and determination of substances present in very low quantities; for example, nanogram quantities of substances in biological fluids, such as urine and serum. A wide variety of enzymes may be used to form the conjugate, but the enzymes selected are often those enzymes which can be detected with great sensitivity. The macromolecular portion of the conjugate can be derived from a wide variety of amino-containing compounds, including, but not limited to, nucleic acids, proteins, hormones, antigens and allergens, which are characterised by containing amino groups.

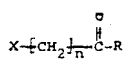
Enzyme conjugates are prepared in a conjugation reaction with a polyfunctional coupling reagent which links the enzyme and macromolecule together by reaction with one or more of the reactive groups in the reactants. In the preparation of enzyme conjugates, it is most desirable to produce enzyme conjugates of high stability, high specificity and good reproducibility.

In some coupling reactions to prepare enzyme conjugates, it has been suggested to employ a conditioner compound, such as a polyamine, to improve specificity of the enzyme conjugate, with resulting improvement in the detection method due to low signal-to-noise ratio during detection in the immunochemical test. The preparation of enzyme conjugates, employing coupling reagents with conditioners, is described in United States Patent No. 4,002,532.

A new coupling agent has been described for preparation of an enzyme-coupled insulin conjugate for use in the immuno-assay of insulin. The coupling agent is meta-maleimidobenzoyl N-hydroxysuccinimide ester (MBS), a bifunctional reagent which acylates the amino groups of the insulin by reaction with the N-hydroxysuccinimide ester group and by forming thioester bonds with the enzyme by addition of the thiol groups to the maleimide group. This coupling agent has been employed in preparing an enzymatically active and immuno-reactive B-D- galactosidase - MBS - insulin conjugate (see *J. Biochem.*, 79, 233-236 (1976), "Enzyme Coupled Immunoassy of Insulin Using a Novel Coupling Reagent", Kitagawa, T and Aikawa, T.). Although the MBS coupling reagent is satisfactory in some respects, it is desirable to obtain enzyme conjugates of greater stability and greater specificity and sensitivity.

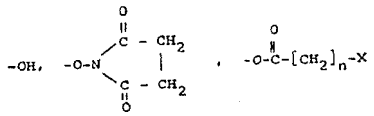
This invention relates to novel coupling reagents, which reagents are useful in the preparation of stable enzyme conjugates, to the method of preparing enzyme conjugates employing the coupling reagents, and to the use of the enzyme conjugates in immunoassay methods.

According to the invention there is provided a method of preparing an enzyme conjugate, which method comprises reacting an amino-containing macromolecule with a coupling reagent of the formula:

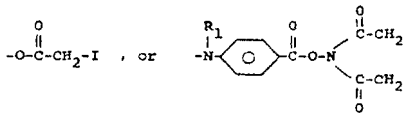


wherein X is halogen; n is a whole number or from 1 to 8; and R represents any radical capable of reacting with an amino group of the macromolecule to form a reagent-macromolecule compound, and reacting the sulph - hydryl - reacting halogen X of the reagent-macromolecule compound with a sulph-hydryl group of an enzyme to form an enzyme-conjugate compound comprising enzyme and macromolecule conjugately linked by the coupling reagent.

X preferably is iodine; n preferably is 1 to 4; and R preferably is



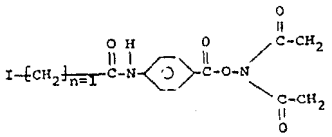
where n and X are as previously defined, particularly an iodoacetyl group



wherein R_1 is hydrogen or alkyl, such as methyl.

Other amino-reacting groups may be employed as the R radical.

The most preferred coupling reagent is N-succinimidyl (4-iodoactyl) aminobenzoate (SIAB) having the structural formula:



The SIAB reagent produces enzymes conjugates with unexpectedly high yield and specific immunochemo, enzyme and conjugate activity. Enzyme conjugates prepared with SIAB, in comparison to other prior art coupling reagents, such as MBS, provide enzyme conjugates of high stability and high specific conjugate yields.

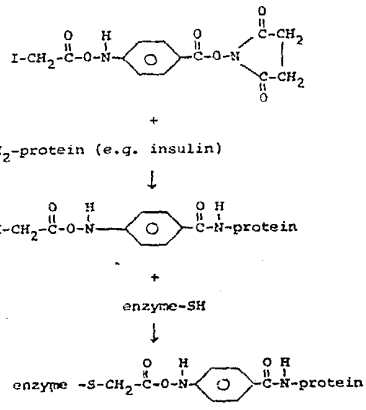
Specific useful coupling reagents include, but are not limited to: iodoacetic acid; iodoacetic anhydride; and N-hydroxysuccinimide ester of iodoacetic acid.

Iodoacetic acid reacts with $-NH_2$ groups in the presence of a water-soluble carbodiimide, such as 1 - ethyl - 3 - (3 - dimethylaminopropyl) carbodiimide. Iodoacetic anhydride reacts directly with $-NH_2$ groups. In other cases, the coupling reagents can be prepared by reacting the selected halo organic acid or acid anhydride in a solvent with the R amino-reacting portion of the molecule, and recovering the reagent. For example, SIAB can be synthesised by reaction of iodoacetic anhydride with *para*-aminobenzoic acid in an organic solvent, such as dioxane. The resultant intermediate compound is

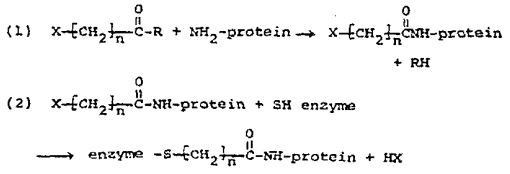
crystallised and the active ester is formed between the intermediate and N - hydroxysuccinimide, with a carbodiimide in an organic solvent; for example, with dicyclohexylcarbodiimide in tetrahydrofuran. The resultant product, which may be crystallised, is SIAB.

The enzyme conjugates are prepared by a conjugation reaction between the amino-reacting subtituent of the coupling reagent and the macromolecule, and, thereafter, reacting the halogen X of the reagent-macromolecule compound with the sulph-hydryl groups of the enzyme. It is essential in the preparation of the enzyme conjugates that the reaction sequence be, firstly, the reaction of the R amino-reacting group, such as the succinimidyl group, with the amino group of the protein or other macromolecule, and, thereafter, reaction of X with sulph-hydryl groups of the enzyme; otherwise, the R group would react with the amino group of the enzyme, resulting in lower yields and a lack of specificity in the resulting enzyme conjugate product so prepared.

For example, the method of the invention provides for the preparation of an enzyme conjugate with a high degree of specific coupling, as represented by the following illustrative reaction with SIAB:



Generically the method of preparation can be represented as follows:—



Typically SIAB is reacted in an aqueous solution with amino groups of the macromolecules under mild conditions. For example, SIAB is reacted with immunoglobulin in 0.05 M phosphate buffer, pH 7.0. The resultant immunoglobulin derivative is then reacted with an enzyme which contains sulph-hydryl groups. One such enzyme is Beta D galactosidase (BG) isolated from *E. coli*. BG is reacted with the derivatised immunoglobulin under mild aqueous conditions. The resulting BG macromolecule conjugates so prepared have a high degree of enzyme activity, immunological specificity, and are obtained in extremely high yields; for example, virtually all the immunoglobulin or other macromolecule and BG are stoichiometrically conjugated to each other. The yield of BG conjugate so achieved is surprisingly high. The conjugates of BG with either antigens or antibodies produced with SIAB are useful in enzyme immunoassays for various antigens or antibodies, including hepatitis, serum proteins, hormones and drugs.

Any SH-reactive enzyme may be used in the method of the invention, such as, for example, acid and alkaline phosphatases, alcohol dehydrogenase catalase, glucose and galactose oxidases, α- and β-galactosidases, lactate dehydrogenase, lysozyme, luciferase, and peroxidases, ribonuclease, rodhanase and esterases.

Useful NH_2-reactive macromolecules includes deoxy and ribonucleic acids, viral proteins, allergens, immunoglobulins, blood group substances, transplantation antigens, carcino embryonic antigen, alpha-fetoprotein and other tumor specific antigens, growth hormone and other polypeptide hormones.

The SIAB is particularly useful in the preparation of immunoglobulin-bacterial enzyme B-D-galactosidase conjugates. The enzyme conjugates produced in accordance with the invention can be used for the detection of antigens, antibodies, allergens, hormones and other macromolecules in low nanogram, or less, quantities by immunoassay techniques. The enzyme conjugates find particular use in those test experiments and detection techinques relating to macromolecules, particularly to the detection and determination of nanogram quantities of substances in biological fluids, such as the detection of antibodies, antigens, allergens and hormones. In particular SIAB enzyme conjugates find use in enzyme immunoassay techinques for hepatitis. In prior art techniques, repeated washings of the patient's serum on the solid support, that is, an antigen disc, are required to remove nonspecific, absorbed serum proteins. However, the use of SIAB conjugates enzymes makes feasible a one-step enzyme immunoassay method. Limited washing or no washing is possible with SIAB enzyme conjugates, since all or substantially all of the specific-form enzyme is bound to the antibody.

The enzyme conjugates may be employed in the prior art techniques for the detection of antibodies; for example, as set forth in U.S. Patent No. 4,002,532 wherein the detection method comprises adhering, such as by absorption, to a solid support or absorbent material, such as a disc, which binds or absorbs the antibodies or other macromolecules to be detected; adding to the solid support material a fluid, such as serum or urine, to bind the antibodies therein to the support material used; for example, incubating the serum with the disc; washing the support material to remove unbound components of the fluid, such as nonspecifically bound and absorbed serum proteins, from the disc surface; adding the enzyme conjugate to the washed support, such as by incubation of the disc with an

enzyme-active SIAB protein conjugate, so that the specific enzyme conjugate is bound to the support in proportion to the quantity of disc-bound antibodies, and thus is a measure of the patient's IgE or other macromolecules to be quantitated; optionally washing the support to remove unbound unspecific enzyme conjugate from the support disc; and determining the enzymatic activity of the bound enzyme conjugate as a measure of the amount of bound antibodies.

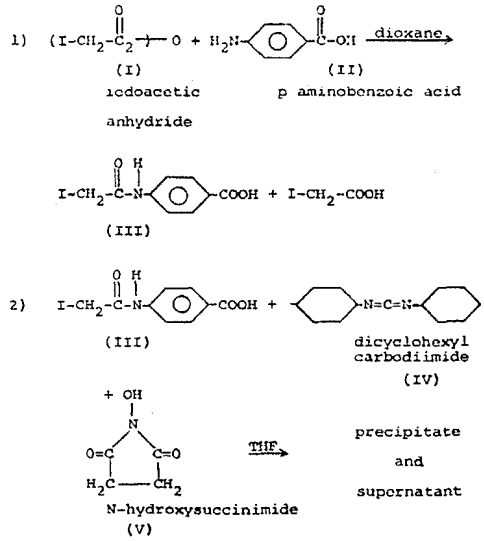
In such a detection method, the high specific yield of the enzyme conjugate provides for a high degree of coupling with the support material in comparison to prior art techinques, as indicated, for example, in the unexpectedly high signal-to-noise ratio in the detection method; that is, the enzyme conjugates of the invention increase sensitivity and specificity by specific bonding of the enzyme conjugate to the solid support, and permit increased sensitivity in the detection of antibodies with lower quantities of enzyme conjugates and in the presence of interfering substances not possible in the prior art enzyme conjugates. For example, when employing the SIAB coupling reagent with a human serum containing a known amount of antigen, the enzyme conjugate is quite stable, and a determination of the amount of bound enzyme is about three and one-half times more than with the MBS coupling reagent at about one-half the amount of enzyme to provide a specific conjugate with an improvement of about 15 fold.

The invention will now be illustrated by specific and preferred Examples.

EXAMPLE 1

Preparation of SIAB Coupling Reagent

N-succinimidyl (4 - iodoacetyl) aminobenzoate (SIAB), the preferred coupling reagent of the invention, was prepared by the following reaction:



354 mg of cpd I (10^3 μmoles) were dissolved in 5 ml dioxane and added to 68.6 mg of cpd II (500 μmoles) in 2.5 ml of dioxane and were reacted for 5 hours at room temperature (20-25°C) in the dark and then at 4°C for 2 days. A white flocculent precipitate (cpd III) was isolated by centrifugation and triturated with ether (0.5 ml) three times. The resulting white powder was dried with hot air with a yield of 160 mg. Cpd IV (86.2 mg; 4×10^4 moles) was added to a solution of cpd III (128 mg; 4×4^{-4} moles) and Cpd V (48.5 mg; 4×10^{-4} moles) and reacted in tetrahydrofuran THF (3.35 ml) at 4°C for 20 hours. A precipitate was removed and the supernatant liquid was recovered and evaporated to dryness and triturated with ether, and pale yellow crystals were recovered of impure SIAB (Cpd VI), yield 135 mg (79.5% yield), m.p. 172-175°C. The impure Cpd VI was recrystallised from methyl alcohol and was washed twice with diethyl ether to provide white crystals of SIAB having an m.p. of 194 to 196°C. (decomp.). Confirmation of this SIAB composition was made by elemental analysis.

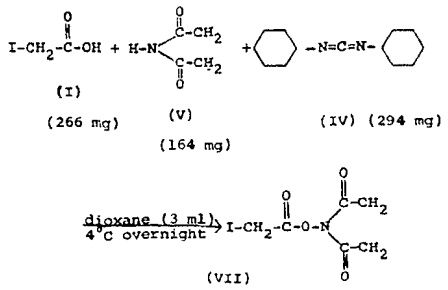
EXAMPLE 2

Preparation of SIAB Enzyme Conjugates

Rabbit antibodies against sheep immunoglobulin (RaShIg) were conjugated to beta - D - galactosidase (BG) with SIAB to provide an enzyme antibody conjugate. The purified RaShIg and the BG were obtained as described in Example 1 of United States Patent No. 4,002,532. A mixture of RaShIg (3.9 mg, 2.63×10^{-8} moles) and SIAB (30λ of 3.6 mg/ml of THF) was prepared (SIAB was added to RaShIg) in an aqueous 0.05 M sodium - phosphate - buffered saline solution (pH 7.0) and the mixture was permitted to react at room temperature overnight; that is, 12 hours in the dark, to provide an acylated RaShIg product, with some of the amino groups of the antibodies reacted with the active succinimidyl group of the SIAB coupling reagent. The reaction was quenched by the addition of glycine (30λ, 4.5×10^{-2} moles) to the acylated RaShIg for 3 hours at room temperature in the dark. To the quenched RaShIg was added BG (25 x molar excess of RaShIg over BG), with the solution adjusted to a pH of 7.8 at 4°C for 2 days. Thereafter, the reaction of the iodine radical with the sulph-hydryl of the enzyme BG was quenched by the addition of 2 - mercapto - ethanol (4×10^{-3} M), and the mixture was maintained at room temperature for 3 hours. The RaShIg-BG conjugate so obtained was diluted, clarified and recovered. There was no detectable loss in activity on enzyme as a result of the coupling procedure.

EXAMPLE 3

Preparation of Iodoacetyl N-Succinimide (INS)



EXAMPLE 4

Comparison of RaShIg-BG Conjugates

A conjugate prepared as in Example 2 was prepared in a buffered enzyme solution of standard units (10,000) of enzyme activity per ml and tested, as in United States Patent No. 4,002,532 (column 5, line 55, column 6, line 1), to determine that the conjugate exhibited a high degree of specificity as illustrated by the S/N ratio shown in the table below, when compared with conjugates prepared in a similar manner, but with prior-art coupling agents MBS and W-R agent (see United States Patent No. 4,002,532, Example 1).

TABLE I

Comparison of S/N of Sheep Immunoglobulin (ShIg) *Conjugates of Beta Galactosidase (BG)*

Units of ShIg-BG Added Per Disc	*Coupling Reagent*		
	SIAB*	MBS*	W-R
1	94±7	41±1	67±4
10	63±2	58±1	52±2
100	32±2	18±0	20±1

* Numbers are S/N ± standard deviation of duplicate measurements. S is binding to immuno specific RaShIg discs. N is binding to normal nonspecific Rabbit RIg discs.

The maximum S/N ratio for SIAB is almost twice the S/N for the MBS coupling agent, and the S/N for SIAB was rising monotonically while the MBS S/N went through a maximum value. Thus, SIAB conjugates could be used at even lower than unit concentrations to provide even better S/N ratios. The S/N ratios establish that the SIAB conjugates are highly specific in comparison to prior-art conjugates. This is borne out by a comparison of SIAB and W-R conjugates in a test for HB_sAg (Table II).

TABLE II

Comparison of Conjugates* of Beta Galactosidase *(BG) in a Test for Hepatitis B-Surface Angiten (HB_sAg)*

HB_sAg (ng)	SIAB**	W-R**	SIAB/W-R
0	0	0	—
0	0	0	—
3.13	0.00462	0.00115	4.02
3.13	0.00322	0.00196	1.64
6.25	0.00815	0.00226	3.61
6.25	0.00645	0.00156	4.13
12.50	0.01435	0.00786	1.83
12.50	0.01275	0.00766	1.66
25.00	0.02035	0.01376	1.55
25.00	0.02145	0.01396	1.54

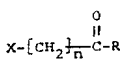
* Conjugates are Rabbit anti-goat Ig coupled to beta galactosidase used to detect goat anti-HB_sAg.

** Numbers are units of BG bound to each immunosorbent disc.

The average SIAB/W-R is 2.5; that is, an average of 2.5 times more SIAB conjugate was specifically bound. The procedure followed was that set forth in United States Patent No. 4,002,532.

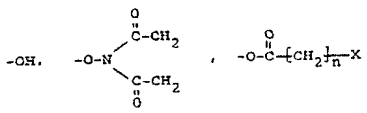
CLAIMS

1. A method of preparing an enzyme conjugate, which method comprises reacting an amino-containing macromolecule with a coupling reagent of the formula:

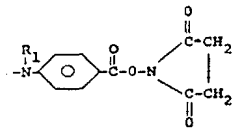


wherein X is halogen; n is a whole number of from 1 to 8; and R represents any radical capable of reacting with an amino group of the macromolecule to form a reagent-macromolecule compound, and reacting the sulph - hydryl - reacting halogen X of the reagent-macromolecule compound with sulph-hydryl group of an enzyme to form an enzyme-conjugate compound comprising enzyme and macromolecule conjugately linked by the coupling reagent.

2. A method according to claim 1 wherein R represents



wherein n and X are as defined in claim 1 or



wherein R_1 is hydrogen or an alkyl group.

3. A method according to claim 1 or 2 wherein X is iodine.

4. A method according to claim 1 or 2 wherein n is from 1 to 4.

5. A method according to claim 1 wherein a haloacetyl N-succinimidyl compound is used as coupling reagent.

6. A method according to claim 1 wherein N-succinimidyl (4-iodoacetyl) aminobenzoate is used as coupling reagent.

7. A method according to any one of the preceding claims wherein the amino-containing macromolecule comprises a non-enzyme proteinaceous macromolecule selected from antibodies, antigens, allergens, hormones, immunoglobulin and serum substances.

8. A method according to any one of the preceding claims wherein the enzyme comprises beta - D - glactosidase.

9. A method according to any one of claims 1 to 6 wherein the enzyme comprises a B-D-galactosidase and the macromolecule comprises immunoglobulin.

10. A method according to claim 1 substantially as described in Example 2.

11. A process for the detection of antibodies, which process comprises:

a) removing the unbound components of the fluid from the solid support material;

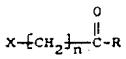
b) adding an enzyme conjugate prepared in accordance with a method as claimed in any one of claims 1 to 10 to the solid support material, whereby the conjugate is bound in proportion to the quantity of bound antibodies to the support material;

c) removing unbound conjugate from the support material; and

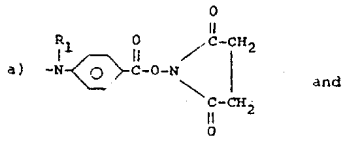
d) determining the presence of bound antibodies by the enzymatic activity of the conjugate.

12. A process according to claim 11 substantially as described with reference to Example 4.

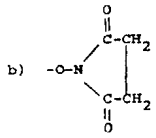
13. A compound suitable for use as a coupling reagent to form enzyme conjugates, which compound is of the formula:



wherein X is halogen; n is a whole number of from 1 to 8; and R is an amino-reactive radical selected from:



and



wherein R_1 is hydrogen or lower alkyl.

14. A compound according to claim 13, which compound is N-succinimidyl (4-iodoacetyl) aminobenzoate.

15. A compound according to claim 13, which compound is iodacetyl - N - succinimide.

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